



Rapid Communication

Exhibit G

Sarcolemmal and Mitochondrial K_{ATP} Channels Mediate Cardioprotection in Chronically Hypoxic Hearts

Xiangrong Kong¹, James S. Tweddell^{2,4}, Garrett J. Gross³ and John E. Baker^{1,3}

Divisions of ¹Pediatric Surgery and ²Cardiothoracic Surgery, ³Department of Pharmacology & Toxicology, Medical College of Wisconsin, Milwaukee, WI, USA and ⁴Section of Cardiothoracic Surgery, Children's Hospital of Wisconsin, Milwaukee, WI 53226, USA

(Received 29 January 2001, accepted in revised form 9 February 2001, published electronically 9 March 2001)

X. KONG, J. S. TWEDDELL, G. J. GROSS AND J. E. BAKER. Sarcolemmal and Mitochondrial K_{ATP} Channels Mediate Cardioprotection in Chronically Hypoxic Hearts. *Journal of Molecular and Cellular Cardiology* (2001) **33**, 1041–1045. Hypoxia from birth increases the resistance of the isolated neonatal heart to ischemia. We determined if increased resistance to ischemia was due to activation of sarcolemmal or mitochondrial K_{ATP} channels. Rabbits ($n=8$ /group) were raised from birth in a normoxic ($F_2O_2=0.21$) or hypoxic ($F_2O_2=0.12$) environment for 8–10 days and the heart perfused with Krebs–Henseleit bicarbonate buffer. A mitochondrial-selective K_{ATP} channel blocker 5-hydroxydecanoate (5-HD) (300 μ mol/l) or a sarcolemmal-selective K_{ATP} channel blocker HMR 1098 (30 μ mol/l) were added alone or in combination for 20 min prior to a global ischemic period of 30 min, followed by 35 min reperfusion. Recovery of ventricular developed pressure was higher in chronically hypoxic than normoxic hearts. 5-HD and HMR 1098 partially reduced the cardioprotective effect of chronic hypoxia, but had no effect in normoxic hearts. The combination of 5-HD and HMR 1098 abolished the cardioprotective effect of chronic hypoxia. We conclude that both sarcolemmal and mitochondrial K_{ATP} channels contribute to cardioprotection in the chronically hypoxic heart.

© 2001 Academic Press

KEY WORDS: 5-hydroxydecanoate; HMR 1098; K_{ATP} channel; Cardiovascular diseases; Hypoxia.

Introduction

Adaptation of the heart to chronic hypoxia from birth results in increased resistance to ischemia,¹ which is associated with activation of K_{ATP} channels.² Two subtypes of K_{ATP} channels exist: mitochondrial K_{ATP} channels, located in the inner mitochondrial membrane, and the surface K_{ATP} channels located in the sarcolemmal membrane. The cardioprotective effects of chronic hypoxia are abolished by glibenclamide,³ a mitochondrial and sarcolemmal K_{ATP} channel blocker.⁴ Thus, it is not known as to which channel mediates cardioprotection.

Selective openers and blockers of the mitochondrial and sarcolemmal K_{ATP} channels have been

identified. The K_{ATP} channel opener diazoxide is 1000 times more selective for opening mitochondrial than sarcolemmal K_{ATP} channels.⁵ The cardioprotective effect of diazoxide is abolished by the selective mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate (5-HD).⁶ 5-HD also abolishes the cardioprotective effects of preconditioning in immature hearts.⁷ The K_{ATP} channel blocker HMR 1883 and its sodium salt HMR 1098 are selective for the sarcolemmal K_{ATP} channel.⁸ However, HMR 1098 does not block preconditioning⁹ which suggests that the sarcolemmal K_{ATP} channel does not contribute to this form of cardioprotection.

The right ventricle is more resistant to ischemia in both normoxic and chronically hypoxic hearts.³ However, the relative roles of mitochondrial and

Please address all correspondence to: John E. Baker, Division of Pediatric Surgery, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA. Tel: (414) 456-8706; Fax: (414) 453-9700; E-mail: jbaker@mcw.edu

sarcolemmal K_{ATP} channels in mediating resistance to ischemia in the right ventricle are unknown. Thus, our objectives were to determine the contribution of the mitochondrial and sarcolemmal K_{ATP} channels to cardioprotection in left and right ventricle afforded by adaptation of hearts to chronic hypoxia.

Materials and Methods

Creation of hypoxia from birth

Rabbits were raised from birth to 8–10 days of age in a hypoxic ($F_1O_2 = 0.12$) or normoxic ($F_1O_2 = 0.21$) environment as described previously.⁷

Perfusion sequence

We performed the following experiments using eight groups ($n = 8$ /group) to determine whether the mitochondrial or sarcolemmal K_{ATP} channels contribute to cardioprotection in chronically hypoxic hearts. The eight groups were as follows: group 1, normoxic, no intervention; group 2, normoxic, treated with 5-HD alone; group 3, normoxic, treated with HMR 1098 alone; group 4, normoxic, treated with 5-HD plus HMR 1098; group 5, chronically hypoxic, no intervention; group 6, chronically hypoxic, treated with 5-HD alone; group 7, chronically hypoxic, treated with HMR 1098 alone; group 8, chronically hypoxic, treated with 5-HD plus HMR 1098. Immediately after aortic cannulation, hearts were perfused in the Langendorff mode at a constant perfusion pressure of 42 mmHg¹ with balloons placed in left and right ventricles. Biventricular function and coronary flow rate were recorded under steady-state conditions.³ 5-HD (300 μ mol/l) or HMR 1098 (30 μ mol/l) were added alone or in combination for 20 min prior to a global ischemic period of 30 min, followed by 35 min of reperfusion.

Recovery of developed pressure was expressed as a percentage of its pre-drug, pre-ischemic value. Results are expressed as the mean \pm s.d. Statistical analysis was performed by use of repeated measures ANOVA with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a Type I error.⁸ If significant, the Mann-Whitney test was used as a second step to identify which groups were significantly different. After ANOVA the data were analysed for differences related to multiple comparisons.³ Significance was set at $P < 0.05$.

Results

To determine the optimal concentration for 5-HD and HMR 1098 for use in the cardioprotection studies, we performed concentration-response studies for each drug (5-HD: 0–450 μ mol/l, HMR 1098: 0–45 μ mol/l) in chronically hypoxic hearts. In chronically hypoxic hearts both 5-HD and HMR 1098 exhibited a "U"-shaped response profile for recovery of left ventricular developed pressure and drug concentration. The optimal concentrations for reducing the cardioprotective effect of chronic hypoxia with 5-HD and HMR 1098 was 300 μ mol/l and 30 μ mol/l, respectively. In normoxic hearts 300 μ mol/l 5-HD and 30 μ mol/l HMR 1098 did not affect recovery of left ventricular developed pressure compared with drug free controls. These concentrations of 5-HD and HMR 1098 are able to block current through the mitochondrial and sarcolemmal K_{ATP} channels, respectively.⁸

Pre-ischemic function

Cardiac function and the effect of K_{ATP} channel blockers on aerobic function prior to ischemia were determined in immature normoxic and chronically hypoxic hearts (Table 1). 5-HD (300 μ mol/l) did not affect heart rate, coronary flow or developed pressure in left or right ventricle in normoxic hearts. However, in chronically hypoxic hearts 5-HD depressed heart rate slightly without affecting coronary flow or developed pressure in either ventricle. HMR 1098 (30 μ mol/l) did not affect heart rate, coronary flow or developed pressure in left or right ventricle in normoxic hearts. However, in chronically hypoxic hearts, HMR 1098 increased left but not right ventricular developed pressure and did not affect heart rate or coronary flow. The combination of 5-HD (300 μ mol/l) plus HMR 1098 (30 μ mol/l) had no effect on heart rate, coronary flow or left and right ventricular developed pressure in either normoxic or chronically hypoxic hearts.

Post-ischemic function

To determine the effect of chronic hypoxia on resistance to myocardial ischemia, recovery of post-ischemic function was examined in normoxic and hypoxic hearts not subjected to drug intervention. Recovery of left ventricular developed pressure following ischemia was greater in chronically hypoxic hearts ($68 \pm 4\%$) compared with normoxic hearts ($44 \pm 5\%$) (Fig. 1). Recovery of developed pressure

Table 1 Hemodynamic values for each group

Groups	Pre-drug			Post-drug			Reperfusion (35 min)					
	Heart rate (beats/min)	Coronary flow rate (ml/min)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)	Heart rate (beats/min)	Coronary flow rate (ml/min)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)	Heart rate (beats/min)	Coronary flow rate (ml/min)	Left ventricle developed pressure (mmHg)	Right ventricle developed pressure (mmHg)
1. Normoxic, no intervention	225±28	6±2	102±7	33±6	—	—	—	—	210±28	5±1	45±4†	23±6†
2. Normoxic + 5-HD (300 µmol/l)	240±16	6±2	97±10	33±6	225±23	6±2	95±12	32±6	225±28	6±2	40±7†	24±4†
3. Normoxic + HMR 1098 (30 µmol/l)	240±16	7±2	99±7	37±6	236±19	6±2	98±10	36±5	221±22	5±1	42±7†	23±3†
4. Normoxic + 5-HD plus HMR 1098	248±14	6±2	101±5	35±4	248±14	6±2	107±7	36±3	236±19	6±2	45±6†	22±3†
5. Hypoxic, no intervention	221±16	9±2‡	100±8	53±3‡	—	—	—	—	210±23	8±1	67±4‡	43±5‡
6. Hypoxic + 5-HD (300 µmol/l)	236±11	10±2	102±9	51±10	210±23*	9±2	103±11	51±11	210±23	7±2	53±5†	34±6†
7. Hypoxic + HMR 1098 (30 µmol/l)	233±21	10±2	101±7	50±6	229±28	10±2	109±6*	54±7	218±35	7±2	55±4†	34±5†
8. Hypoxic + 5-HD plus HMR 1098	244±11	11±2	103±6	50±5	244±11	11±3	109±7	55±6	229±28	8±3	45±5†	31±7†

Values are means±s.e., from 8 hearts/group. * = $P<0.05$, pre-drug v post-drug; † = $P = 0.05$, pre-drug v reperfusion; ‡ = $P<0.05$, normoxic v hypoxic.

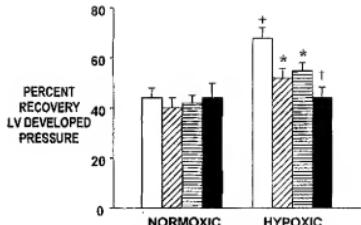


Figure 1 Recovery of left ventricular developed pressure following 20 min treatment with 5-HD alone (300 $\mu\text{mol/l}$), HMR 1098 (30 $\mu\text{mol/l}$) alone and 5-HD (300 $\mu\text{mol/l}$) combined with HMR 1098 (30 $\mu\text{mol/l}$) prior to 30 min global ischemia and 35 min reperfusion. (□), Control; (▨), 5-HD alone; (▨), HMR 1098 alone; (▨), 5-HD combined with HMR 1098. Data are means \pm s.d. ($n=8$ hearts/group). +, $P<0.05$, normoxic v hypoxic; *, $P<0.05$, drug alone v drug-free control; †, $P<0.05$, drug alone v drugs combined.

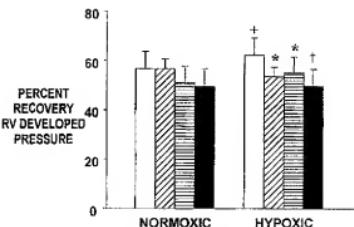


Figure 2 Recovery of right ventricular developed pressure following 20 min treatment with 5-HD alone (300 $\mu\text{mol/l}$), HMR 1098 (30 $\mu\text{mol/l}$) alone and 5-HD (300 $\mu\text{mol/l}$) combined with HMR 1098 (30 $\mu\text{mol/l}$) prior to 30 min global ischemia and 35 min reperfusion. (□), Control; (▨), 5-HD alone; (▨), HMR 1098 alone; (▨), 5-HD combined with HMR 1098. Data are means \pm s.d. ($n=8$ hearts/group). +, $P<0.05$, normoxic v hypoxic; *, $P<0.05$, drug alone v drug-free control; †, drugs combined v drug-free control.

in the right ventricle was greater in chronically hypoxic hearts (78 \pm 8%) compared with normoxic hearts (71 \pm 10%) (Fig. 2).

To determine the effect of blockade of mitochondrial and sarcolemmal K_{ATP} channels upon resistance to myocardial ischemia, recovery of post-ischemic function was measured in normoxic and chronically hypoxic hearts treated with 5-HD and HMR 1098 either alone or in combination prior to ischemia. 5-HD and HMR 1098 alone partially

reduced recovery of left ventricular developed pressure in chronically hypoxic hearts to 52 \pm 4% and 55 \pm 3%, respectively, but had no effect in normoxic hearts (Fig. 1). However, the combination of 5-HD and HMR 1098 completely abolished the cardioprotective effect of chronic hypoxia (68 \pm 4% to 44 \pm 5%) but had no effect in normoxic hearts (Fig. 1). 5-HD and HMR 1098 alone completely abolished the cardioprotective effects of chronic hypoxia in the right ventricle (Fig. 2). The combination of 5-HD and HMR 1098 in chronically hypoxic hearts further depressed recovery of developed pressure in right ventricle to 62 \pm 9% (Fig. 2).

Discussion

Previously we showed that chronic hypoxia from birth increased resistance of isolated hearts to ischemia, and that the cardioprotective effect of hypoxia was abolished by glibenclamide, a non-selective K_{ATP} channel blocker. However, the identity of the K_{ATP} channel subtype associated with increased resistance to ischemia remained unknown. In this report, we show that both mitochondrial and sarcolemmal K_{ATP} channels contribute to the cardioprotective effects of adaptation to chronic hypoxia from birth. The mitochondrial and sarcolemmal K_{ATP} channels did not contribute to cardioprotection in normoxic hearts. Simultaneous inhibition of both sarcolemmal and mitochondrial K_{ATP} channels completely abolished the cardioprotective effects of chronic hypoxia.

Our study is the first to demonstrate that cardioprotection induced by adaptation to chronic hypoxia involves activation of both the sarcolemmal and mitochondrial K_{ATP} channel. In contrast, cardioprotection induced by ischemic preconditioning involves the mitochondrial but not the sarcolemmal K_{ATP} channel.⁹ Similarly, cardioprotection induced by opioids can also be abolished with 5-hydroxydecanoate but not HMR 1098.¹⁰ These studies on cardioprotection induced by ischemic preconditioning and opioids were performed on unstressed normoxic hearts and these hearts may respond differently than those exposed to chronic hypoxia.

Most studies of cardioprotection have investigated resistance to ischemia in the left ventricle. The use of the isolated heart model allows simultaneous measurement of resistance to ischemia in both left and right ventricle and permits comparisons to be made. We showed that the right ventricle was more resistant to ischemia than the left ventricle in both normoxic and chronically

hypoxic hearts. The isolated heart model avoids the systemic effects of K_{ATP} channel openers or blockers. There is very little information available on the role of the K_{ATP} channel in mediating resistance to ischemia in the right ventricle. 5-HD and HMR 1098 were able to abolish the cardioprotective effects of chronic hypoxia in right ventricle indicating that mitochondrial and sarcolemmal K_{ATP} channels mediate resistance to ischemia in the chronically hypoxic right ventricle.

Cardioprotection of the myocardium can be induced by several ways including ischemic preconditioning¹¹ and chronic hypoxia.¹ However, distinct differences are present in the mechanisms underlying cardioprotection by ischemic preconditioning and adaptation to chronic hypoxia. In late preconditioning, nitric oxide generated from the NOS2 isoform protects the heart against sustained ischemia.¹¹ However, our studies with chronic hypoxia show nitric oxide generated from the NOS3 isoform is responsible for protecting the heart against ischemia.¹² Preconditioning is mediated by activation of the mitochondrial K_{ATP} channel.⁹ Our study shows both sarcolemmal and mitochondrial K_{ATP} channels mediate cardioprotection in chronically hypoxic hearts. Thus, the operative mechanisms by which adaptation to chronic hypoxia and late preconditioning protect the heart against ischemia are separate.

We conclude the sarcolemmal and mitochondrial K_{ATP} channels contribute to cardioprotection in the chronically hypoxic heart. Further investigations are needed to clarify the mechanisms by which K_{ATP} channels become active during adaptation to chronic hypoxia and produce an increased resistance to myocardial ischemia.

Acknowledgements

This work was supported in part by grants HL-54075 and HL-08311 from the National Institutes of Health and the Children's Hospital of Wisconsin Foundation.

The authors gratefully acknowledge the secretarial assistance of Mary Lynne Koenig.

References

- BAKER JE, BOERBOOM LE, OLINGER GN, BAKER JE. Tolerance of the developing heart to ischemia: impact of hypoxemia from birth. *Am J Physiol* 1995; 268: H1165-H1173.
- BAKER JE, CONTNEY SJ, GROSS GJ, BORNJAK ZJ. KATP channel activation in a rabbit model of chronic myocardial hypoxia. *J Mol Cell Cardiol* 1997; 29: 845-848.
- BAKER JE, CURRY BD, OLINGER GN, GROSS GJ. Increased tolerance of the chronically hypoxic immature heart to ischemia. Contribution of the KATP channel. *Circulation* 1997; 95: 1278-1285.
- GROVER GJ, GARLID KD. ATP-sensitive potassium channels: a review of their cardioprotective pharmacology. *J Mol Cell Cardiol* 2000; 32: 677-695.
- GARLID KD, PAUCEK P, YAROV-YAROVY V, SUN X, SCRINDLER PA. The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J Biol Chem* 1996; 271: 8796-8799.
- GARLID KD, PAUCEK P, YAROV-YAROVY V, MURRAY HN, DARBBENZIO RB, D'ALONZO AJ, LODGE NJ, SMITH MA, GROVER GJ. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K⁺ channels. Potential mechanism of cardioprotection. *Circ Res* 1997; 81: 1072-1082.
- BAKER JE, HOLMAN P, GROSS GJ. Preconditioning in immature rabbit hearts: role of KATP channels. *Circulation* 1999; 99: 1249-1254.
- SATO T, SASAKI N, SEHARASEYON J, O'ROURKE B, MARBAN E. Selective pharmacologic agents implicate mitochondrial but not sarcolemmal K_{ATP} channels in ischemic cardioprotection. *Circulation* 2000; 101: 2418-2423.
- FAYER RM, EELLS JT, HSU AK, HENRY MM, GROSS GJ. Ischemic preconditioning in rats: role of mitochondrial K_{ATP} channel in preservation of mitochondrial function. *Am J Physiol* 2000; 278: H305-H312.
- FAYER RM, HSU AK, NAGASE H, GROSS GJ. Opioid-induced cardioprotection against myocardial infarction and arrhythmias: mitochondrial versus sarcolemmal ATP-sensitive potassium channels. *J Pharmacol Exp Ther* 2000; 294: 451-457.
- BOLLI R. The late phase of preconditioning. *Circ Res* 2000; 87: 972-983.
- SRI Y, PRITCHARD KA JR, HOLMAN P *et al*. Chronic hypoxia increases constitutive nitric oxide synthase activity: implications for resistance to myocardial ischemia. *Free Radic Biol Med* 2000; 29: 695-703.